EPH-LIKE RECEPTOR PROTEIN TYROSINE KINASES

Field of the Invention

The invention relates generally to receptor protein tyrosine kinases (PTKs) and particularly to novel Eph-like receptor PTKs, to fragments and analogs thereof, and to nucleic acids encoding same. The present invention also relates to methods of producing and using such receptors.

Background of the Invention

Receptor PTKs are a structurally related family of proteins that mediate the response of cells to extracellular signals (Ullrich et al. Cell 61, 203-212 (1990)). These receptors are characterized by three major functional domains: an intracellular region containing the sequences responsible for catalytic activity, a single hydrophobic membrane-spanning domain, and a glycosylated extracellular region whose structure determines ligand binding specificity. Signal transduction is initiated by the binding of growth or differentiation factors to the extracellular domain of their cognate receptors. Ligand binding facilitates dimerization of the receptor which can induce receptor autophosphorylation. Both soluble and membrane-associated protein ligands have been shown to function in this manner. This process is the initial step in a cascade of interactions involving the phosphorylation of a variety of cytoplasmic substrates and culminating in a biological response by the cell. The best characterized response to tyrosine kinase receptor activation is cell growth. However, analysis of the role of some growth factors *in vivo* suggests that differentiation or cell

survival might also be mediated by tyrosine kinase receptor/ligand interactions.

Receptor PTKs have been grouped into fairly well-defined families on the basis of both sequence homology and shared structural motifs. The amino acid sequence of the portion of the intracellular domain responsible for the catalytic activity is well conserved among all tyrosine kinases and even more closely matched within a receptor sub-family. Comparisons of this portion of the amino acid sequence have been used to construct phylogenetic trees depicting the relatedness of family members to each other and to the tyrosine kinases as a whole (Hanks and Quinn, Methods Enzymol. 200, 38-62 (1991)). This sequence conservation has also been exploited in order to isolate new tyrosine kinases using the polymerase chain reaction (PCR) (Wilks, Proc. Natl. Acad. Sci. USA 86, 1603-1607 (1989)). Oligonucleotides based on the highly conserved catalytic domain of PTKs can be used as PCR primers to amplify related sequences present in the template. These fragments can then be used as probes for isolation of the corresponding full-length receptor clones from cDNA libraries. Anti-phosphotyrosine antibodies have also been used to identify PTK cDNA clones in phage expression libraries (Lindberg and Pasquale, Methods Enzymol. 200, 557-564 (1991)). These strategies have been used by a number of investigators to identify an ever-increasing number of protein tyrosine kinase receptors.

There are now 51 distinct PTK receptor genes that have been published and divided into 14 sub-families One such sub-family is the EPH-like receptors. The prototype member, EPH, was isolated by Hirai et.al. (Science 238, 1717-1720 (1987)) using low

stringency hybridization to a probe derived from the viral oncogene v-fps. EPH-like receptors have been implicated in cell growth based in part on studies which show that overexpression of the gene in NIH3T3 cells causes focus formation in soft agar and tumors in nude mice (Maru et al. Oncogene 5, 199-204 (1990)). Other members of the EPH sub-family which have been identified include the following:

ECK (Lindberg et al. Mol. Cell. Biol. 10, 6316-6324 (1990))

Elk (Lhoták et al. Mol. Cell. Biol. 11, 2496-2502 (1991))

Ceks 4,5,6,7,8,9, and 10 (Pasquale, Cell Regulation 2, 523-534 (1991); Sajjadi et al. The New Biologist 3, 769-778 (1991); Sajjadi and Pasquale Oncogene 8, 1807-1813 (1993))

HEK2 (Bohme et al. Oncogene 8, 2857-2862 (1993))

Eek, Erk (Chan and Watt, Oncogene 6, 1057-1061 (1991))

Ehk1, Ehk2 (Maisonpierre et al. Oncogene 8, 3277-3288 (1993))

Homologs for some of these receptors have been identified in other species (Wicks et al. Proc. Natl. Acad. Sci. USA 89, 1611-1615 (1992)); Gilardi-Hebenstreit et al. Oncogene 7, 2499-2506 (1992)). The expression patterns and developmental profiles of several family members suggest that these receptors and their ligands are important for the proliferation, differentiation and maintenance of a variety of tissues (Nieto et al. Development 116, 1137-1150 (1992)). Structurally, EPH sub-family members are characterized by an Ig-like loop, a cysteine rich region, and two fibronectin-type repeats in their extracellular domains. The amino acid sequences of the catalytic domains are

more closely related to the SRC sub-family of cytoplasmic PTKs than to any of the receptor PTKs. Among the catalytic domains of receptor PTKs, the EPH sub-family is most similar in amino acid sequence to the epidermal growth factor receptor sub-family.

It is an object of the invention to identify novel receptors belonging to the EPH sub-family. A directed PCR approach has been used to identify five human EPH-like receptors from a human fetal brain cDNA library. These receptors are designated HEK4, HEK5, HEK7, HEK8, and HEK11. The relationship of these receptors to previously identified EPH-like receptors is as follows:

HEK4 is the human homolog of Cek4 (chicken) and Mek4 (mouse) and is identical to HEK (Boyd et al. J. Biol. Chem. 267, 3262-3267 (1992); Wicks et al., 1992) which was previously isolated from a human lymphoid tumor cell line.

HEK5 is the human homolog of Cek5, a full-length eph-like receptor clone from chicken. A portion of the HEK5 sequence was previously disclosed as ERK, a human clone encoding about sixty amino acids (Chan and Watt, 1991)

HEK7 is the human homolog of Cek7 isolated from chicken.

HEK8 is the human homolog of Cek8 a full-length clone from chicken and Sek, a full-length clone from mouse. (Nieto et al., 1992; Sajjadi et al., 1991)

HEK11 does not have a known non-human homolog. With the addition of the new members HEK5, HEK7, HEK8 and HEK11 and the report of a PCR fragment encoding an eph-like receptor (Lai & Lemke Neuron 6, 691-704 (1991)), a total of twelve distinct sequences that represent EPH-like receptors have be n published, making it the largest known sub-family of PTKs.

It is a further object of the invention to generate soluble EPH-like receptors and antibodies to EPH-like receptors. Soluble receptors and antibodies are useful for modulating EPH-like receptor activation.

Summary of the Invention

The present invention provides novel EPH-like receptor protein tyrosine kinases. More particularly, the invention provides isolated nucleic acids encoding four novel members of the sub-family of EPH-like receptor PTKs which are referred to collectively as HEKs (human-eph like kinases). Also encompassed are nucleic acids which hybridize under stringent conditions to EPH-like receptor nucleic acids. Expression vectors and host cells for the production of receptor polypeptides and methods of producing receptors are also provided.

Isolated polypeptides having amino acid sequences of EPH-like receptors are also provided, as are fragments and analogs thereof. Antibodies specifically binding the polypeptides of the invention are included. Also comprehended by the invention are methods of modulating the endogenous activity of an EPH-like receptor and methods for identifying receptor ligands.

Description of the Figures

Figure 1 shows the nucleotide and predicted amino acid sequence of the HEK5 receptor. (SEQ ID NO: 10 and SEQ ID NO: 11)

Figure 2 shows the nucleotide and predicted amino acid sequence of the HEK7 receptor. (SEQ ID NO 12 and SEQ ID NO:13) (F)

Figure 3 shows the nucleotide and predicted amino acid sequence of the HEK8 receptor. (SEQ ID NO 14 and SEQ ID NO: 15)

Figure 4 shows the nucleotide and predicted amino acid sequence of the HEK11 receptor (SEQ ID NO: 10 and SEQ ID NO: 11) (F)

Figure 5 shows the comparison of the amino acid sequences of the human EPH receptor sub-family. The multiple sequence alignment was done using the LineUp program included in the Genetics Computer Group sequence analysis software package (Genetics Computer Group, (1991), Program Manual for the GCG Package, Version 7, April 1991, Madison, Wisconsin, USA 53711). Dots indicate spaces introduced in order to optimize alignment. The predicted transmembrane domains and signal sequences of each receptor are indicated by underlining and italics, respectively. Cysteine residues conserved throughout the sub-family are indicated with asterisks. Arrows indicate the tyrosine kinase catalytic domain. Amino acid sequences of EPH, ECK and HEK2 were taken from the appropriate literature references.

Figure 6 shows the molecular phylogeny of the EPH sub-family of receptor protein tyrosine kinases. Catalytic domain sequences were analyzed as described by Hanks and Quinn, 1991. The scale bar represents an arbitrary evolutionary difference unit. The EPH branch, which has been shown with a discontinuity for the sake of compactness, is 23.5 units in length.

Figures 7-11 show Northern blot analyses of the tissue distribution of the HEK receptors. Receptor cDNA probes, labeled with ^{32}P, were hybridized to either 2 µg of poly A^+ RNA from human tissues (panel A, Clontech) or 10 µg of total RNA from rat tissues (panel B). Sizes of the transcripts were determined by comparison with RNA molecular weight markers (Bethesda Research Labs,

Gaithersburg, MD). Figure 7, HEK4; Figure 8, HEK5; Figure 9, HEK7; Figure 10; HEK8; Figure 11; HEK 11.

Detailed Description of the Invention

The present invention relates to novel EPH-like receptor protein tyrosine kinases. More particularly, the invention relates to isolated nucleic acids encoding four novel members of the sub-family of EPH-like receptor PTKs. These four members are designated herein as HEK (human eph-like kinases). Nucleic acids encoding HEK receptors were identified in a human fetal brain cDNA library using oligonucleotide probes to conserved regions of receptor PTKs and EPH-like receptor PTKs. The predicted amino acid sequences of three HEK receptors had extensive homology in the catalytic domain to previously identified EPH-like receptors Cek5, Cek7 and Cek8 isolated from chicken and, accordingly, are designated HEK5, HEK7 and HEK8. The predicted amino acid sequence of the fourth HEK receptor revealed that it was not a homolog of any previously identified EPH-like receptor. It is designated HEK11. It is understood that the term "HEKs" comprises HEK5, HEK7, HEK8 and HEK11 as well as analogs, variants, and mutants thereof which fall within the scope of the invention.

The invention encompasses isolated nucleic acids selected from the group consisting of:

(a) the nucleic acids set forth in any of SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, or SEQ ID NO: 16 and their complementary strands;

(b) a nucleic acid hybridizing to the coding regions of the nucleic acids in any of SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, or SEQ ID NO: 16 under stringent conditions; and

(c) a nucleic acid of (b) which, but for the degeneracy of the genetic code, would hybridize to the coding regions of the nucleic acids in any of SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, or SEQ ID NO: 16. The nucleic acids of the invention preferably hybridize to HEK5, HEK7, HEK8, or HEK11 coding regions under conditions allowing up to about 5% nucleotide mismatch based upon observed nucleic acid identities among known human or nonhuman EPH-like receptors. An example of such a condition is hybridization at 60° in 1M Na^+ followed by washing at 60° in 0.2XSSC. Other hybridization conditions may be ascertained by one skilled in the art which allow base pairing with similar levels of mismatch.

In a preferred embodiment, the isolated nucleic acids encode polypeptides having the amino acid sequences of HEK5, HEK7, HEK8 or HEK11. A nucleic acid includes cDNA, genomic DNA, synthetic DNA or RNA. Nucleic acids of this invention may encode full-length receptor polypeptides having an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic domain, or may encode fragments such as extracellular domains which are produced in a soluble, secreted form. Nucleic acid constructs which produce soluble HEK receptors are described in Example 3. Polypeptides and fragments encoded by the nucleic acids have at least one of the biological activities of an EPH-like receptor protein tyrosine kinase, such as the ability to bind ligand.

The invention also encompasses nucleic acids encoding chimeric proteins wherein said proteins comprise part of the amino acid sequence of a HEK receptor linked to an amino acid sequence from a heterologous protein. One example of such a chimeric protein is an extracellular domain of a HEK receptor

fused to a heterologous receptor cytoplasmic domain. Example 5 describes the construction and expression of a chimeric receptor comprising the HEK8 extracellular domain with the trkB cytoplasmic domain and a second chimeric receptor comprising the HEK11 extracellular domain with the trkB cytoplasmic domain. HEK receptors may also be fused to other functional protein domains, such as an Ig domain which acts as an antibody recognition site.

The nucleic acids of the present invention may be linked to heterologous nucleic acids which provide expression of receptor PTKs. Such heterologous nucleic acids include biologically functional plasmids or viral vectors which provide genetic elements for transcription, translation, amplification, secretion, etc. One example of an expression vector suitable for producing EPH-like receptors of the present invention is pDSRα which is described in Example 3. It is understood that other vectors are also suitable for expression of EPH-like receptors in mammalian, yeast, insect or bacterial cells. In addition, in vivo expression of nucleic acids encoding EPH-like receptor PTKs is also encompassed. For example, tissue-specific expression of EPH-like receptors in transgenic animals may be readily effected using vectors which are functional in selected tissues.

Host cells for the expression of EPH-like receptor PTKs will preferably be established mammalian cell lines, such as Chinese Hamster Ovary (CHO) cells or NIH 3T3 cells, although other cell lines suitable for expression of mammalian genes are readily available and may also be used. Such host cells are transformed or transfected with nucleic acid constructs suitable for expression of an EPH-like receptor. Transformed or

transfected host cells may be used to produce suitable quantities of receptor for diagnostic or therapeutic uses and to effect targeted expression of EPH-like receptors in selected adult tissues, such as brain, kidney, and liver, or in embryonic or rapidly dividing tissues.

The present invention provides purified and isolated polypeptides having at least one of the biological properties of an EPH-like receptor (e.g. ligand binding, signal transduction). The isolated polypeptides will preferably have an amino acid sequence as shown in any of SEQ ID NO: [10], SEQ ID NO: [12], SEQ ID NO: [14] or SEQ ID NO: [16]. Polypeptides of this invention may be full-length polypeptides having an extracellular domain, a transmembrane domain, and a cytoplasmic domain, or may be fragments thereof, e.g., those having only an extracellular domain or a portion thereof. It will be understood that the receptor polypeptides may also be analogs or naturally-occurring variants of the amino acid sequences shown in SEQ ID NO: [10], SEQ ID NO: [12], SEQ ID NO: [14] or SEQ ID NO: [16]. Such analogs are generated by amino acid substitutions, deletions and/or insertions using methods available in the art.

Polypeptides of the invention are preferably the product of expression of an exogenous DNA sequences, i.e., EPH-like receptors are preferably produced by recombinant means. Methods of producing EPH-like receptors comprising culturing host cells which have been transformed or transfected with vectors expressing an EPH-like receptor are also encompassed. EPH-like receptors, particularly fragments, may also be produced by chemical synthesis. The polypeptides so produced may be glycosylated or nonglycosylated depending upon the host cell employed, or may have a methionine residue at the amino terminal end. The polypeptides so produced

are identified and recovered from cell cultures employing methods which are conventional in the art.

EPH-like receptors of the present invention are used for the production of antibodies to the receptors. Antibodies to HEK receptors have been described in Example 4. Antibodies which recognize the polypeptides of the invention may be polyclonal or monoclonal and may be binding fragments or chimeric antibodies. Such antibodies are useful in the detection of EPH-like receptors in diagnostic assays in the purification of receptor, and in the modulation of EPH-like receptor activation.

As described in co-pending and co-owned U.S. Serial No. 08/145,616, the only known ligand for an EPH-like receptor is a protein which binds to and induces phosphorylation of the eck receptor. The ECK receptor ligand was previously identified as B61. (Holzman et al. Mol. Cell. Biol. 10, 5830-5838 (1990)). The availability of ECK receptor was important for the identification of a ligand since B61, although known, had not been previously implicated as an ECK receptor ligand. Therefore, EPH-like receptors having ligand binding domains are useful for the identification and purification of ligands. Polypeptides of the present invention may be used to identify and purify ligands for HEK5, HEK7, HEK8 and HEK11 receptors. Binding assays for the detection of potential ligands may be carried out in solution or by receptor immobilization on a solid support using methods such as those described in co-pending and co-owned U.S. Serial No. 08/145,616. Such assays may employ an isolated ligand binding domain of a HEK receptor. Alternatively, a HEK ligand binding domain fused to an Ig domain may be used to detect the presence of HEK ligand on cell surfaces.

Soluble EPH-like receptors may be used to modulate (i.e., increase or decrease) the activation of the cell-associated receptors, typically by competing with the receptor for unbound ligand. Modulation of EPH-like receptor activation may in turn alter the proliferation and/or differentiation of receptor-bearing cells. For example, based upon the observed tissue distribution of the receptors of this invention (see Table 5), soluble HEK7 receptor is likely to primarily affect proliferation and/or differentiation of brain cells, while soluble HEK5 receptor may affect primarily brain and pancreatic cells, although effects of HEK5 receptor on other tissues may not be excluded.

Antibodies to EPH-like receptors are useful reagents for the detection of receptors in different cell types using immunoassays conventional to the art. Antibodies are also useful therapeutic agents for modulating receptor activation. Antibodies may bind to the receptor so as to directly or indirectly block ligand binding and thereby act as an antagonist of receptor activation. Alternatively, antibodies may act as an agonist by binding to receptor so as to faciliate ligand binding and bring about receptor activation at lower ligand concentrations. In addition, antibodies of the present invention may themselves act as a ligands by inducing receptor activation. It is also contemplated that antibodies to EPH-like receptors are useful for selection of cell populations enriched for EPH-like receptor bearing cells. Such populations may be useful in cellular therapy regimens where it is necessary to treat patients which are depleted for certain cell types.

The isolated nucleic acids of the present inventions may be used in hybridization assays for the detection and quantitation of DNA and/or RNA coding for HEK5, HEK7, HEK8, HEK11 and related receptors. Such

assays are important in determining the potential of various cell types to express these receptors and in determining actual expression levels of HEK receptors. In addition, the nucleic acids are useful for detecting abnormalities in HEK receptor genes, such as translocations, rearrangements, duplications, etc.

Therapeutic regimens involving EPH-like receptors will typically involve use of the soluble form of the receptor contained in a pharmaceutical composition. Such pharmaecutical compositions may contain pharmaceutically acceptable carrier, diluents, fillers, salts, buffers, stabilizers and/or other materials well known in the art. Further examples of such constituents are described in Remington's Pharmaceutical Sciences 18th ed., A.R. Gennaro, ed. (1990). Administration of soluble EPH-like receptor compositions may be by a variety of routes depending upon the condition being treated, although typically administration will occur by intravenous or subcutaneous methods. Pharmaceutical compositions containing antibodies to EPH-like receptors will preferably include mouse-human chimeric antibodies or CDR-grafted antibodies in order to minimize the potential for an immune response by the patient to antibodies raised in mice. Other components of anti-EPH antibody compositions will be similar to those described for soluble receptor.

The amount of soluble Eph-like receptors or anti-Eph antibody in a pharmaceutical composition will depend upon the nature and severity of the condition being treated. Said amount may be determined for a given patient by one skilled in the art. It is contemplated that the pharmaceutical compositions of the present invention will contain about 0.01 µg to about

100 mg of soluble receptor or anti-Eph antibody per kg body weight.

A method for modulating the activation of an EPH-like receptor PTK is also provided by the invention. In practicing this method, a therapeutically effective amount of a soluble EPH-like receptor or an anti-EPH antibody is administered. The term "therapeutically effective amount" is that amount which effects an increase or decrease in the activation of an EPH-like receptor and will range from about 0.01 μg to about 100 mg of soluble receptor or anti-EPH antibody per kg body weight. In general, therapy will be appropriate for a patient having a condition treatable by soluble receptor or anti-EPH antibody and it is contemplated that such a condition will in part be related to the state of proliferation and/or differentiation of receptor-bearing cells. Based upon the tissue distribution of HEK receptors shown in Table 4, treatment with the pharmaceutical compositions of the invention may be particularly indicated for disorders involving brain, heart, muscle, lung, or pancreas. However, some HEK receptors are displayed on a wide variety of tissues, so it is understood that the effects of modulating receptor activation may not be limited to those tissues described herein.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof. Recombinant DNA methods used in the following examples are generally as described in Sambrook et al. Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, 2nd ed. (1989)

EXAMPLE 1

Cloning and Sequencing of *HEK* Receptor cDNA

We have isolated clones for five members of the EPH sub-family of receptor PTKs from a human fetal brain cDNA library. Oligonucleotides were designed based on conserved amino acid sequences within the kinase domain. Primer I was based on the amino acid sequence Trp-Thr-Ala-Pro-Glu-Ala-Ile (SEQ ID NO: 1), which is well-conserved among PTKs of many families. Primer II was based on the sequence Val-Cys-Lys-Val-Ser-Asp-Phe-Gly (SEQ ID NO: 2), which is invariant among EPH sub-family members but, except for the sequence Asp-Phe-Gly, is rarely found in other PTKs. Fully degenerate oligonucleotides corresponding to reverse translations of these protein sequences were synthesized and utilized as primers in a polymerase chain reaction (PCR) with disrupted phage from a human fetal brain cDNA library as the template. The products of this PCR reaction were cloned into the plasmid vector pUC19 and the nucleotide sequence of the inserts was determined. Of the 35 PCR inserts sequenced, 27 were recognizable as portions of PTK genes. Their correspondence to previously published sequences is summarized in Table 1.

TABLE 1

Receptor	PCR Products		Number of Clones
Elk	VCKVSDFGLSRYLQDDTSDPTYTSSLGGKIPVRWTAPEAI	(SEQ ID NO: 3)	2
HEK4, HEK7	VCKVSDFGLSRVLEDDPEAAYTT RGGKIPIRWTAPEAI	(SEQ ID NO: 4)	5*
HEK5	VCKVSDFGLSRFLEDDTSDPTYTSALGGKIPIRWTAPEAI	(SEQ ID NO: 5)	8
HEK8	VCKVSDFGMSRVLEDDPEAAYTT RGGKIPIRWTAPEAI	(SEQ ID NO: 6)	4
HEK11	VCKVSDFGLSRVIEDDPEAVYTTT GGKIPVRWTAPEAI	(SEQ ID NO: 7)	1
SRC	VCKVSDFGLAR LIEDNEYTARQ GAKFPIKWTAPEAI	(SEQ ID NO: 8)	6*
PDGF-β	VCKVSDFGLARDIMRDSNYISK GSTFLPLKWTAPEAI	(SEQ ID NO: 9)	1

An asterisk indicates that different nucleic acid sequences encoded the amino acid sequence shown.

Six PCR inserts predict amino acid sequences which are identical to a portion of SRC, although they comprise two distinct nucleotide sequences. One insert appears to code for the human platelet derived growth factor (PDGF)-β receptor. The remaining 18 PCR inserts consist of 6 distinct nucleotide sequences, all of which appear to be fragments of EPH sub-family members. One of the sequence predicts an amino acid sequence identical to the corresponding region of rat Elk (Lhotak et al., 1991)) and is likely to represent its human homolog. Two inserts predict amino acid sequences which match the translation of the PCR fragment tyro-4 (Lai and Lemke, 1991)) but are clearly distinct at the nucleotide level while two others correspond to tyro-1 and tyro-5. The sixth PCR insert has a previously unreported *EPH*-related sequence. Since five of the clones contained portions of potential EPH sub-family members for which full-length sequences had not been reported, each was radiolabeled and used as a probe to screen a human fetal brain cDNA library. Several clones corresponding to each of the five probes were isolated. For each of the five receptors, the nucleotide sequence of the clone containing the largest portion of the predicted coding region was determined.

A single cDNA clone containing the complete coding region was isolated only for *HEK4*. The portions of *HEK5, HEK7, HEK10 and HEK11* coding for the amino terminus of these receptors were not found in any of the clones. In order to obtain the complete coding sequence, the Rapid Amplification of cDNA Ends (RACE) technique was employed. In some cases, more than one round of RACE was necessary to obtain the missing portion of the coding region. Using this strategy, complete coding sequences were obtained for all clones except *HEK7* which lacked the complete leader sequence.

The DNA sequences of *HEK5*, *HEK7*, *HEK8* and *HEK11* are shown in Figures 1-4, respectively, and in SEQ ID NO: 10 (HEK5), SEQ ID NO: 12 (HEK7), SEQ ID NO: 14 (HEK 8) and SEQ ID NO: 16 (HEK11). The amino acid sequences are shown in SEQ ID NO: 11 (HEK5), SEQ ID NO: 13 (HEK7), SEQ ID NO: 15 (HEK8) and SEQ ID NO: 17 (HEK 11).

EXAMPLE 2

Analysis of HEK Receptor Sequences

HEK5, HEK7, HEK8 and HEK11 represent novel human EPH sub-family members, although homologs for all except HEK11 have been isolated from other species. We refer to human EPH receptor sub-family members as HEKs (human EPH-like kinases) following the nomenclature of Wicks et al., 1992). We have chosen names and numbers for these receptors to correspond with previously discovered members of the family in chicken (Ceks) and in mouse (Mek) (Sajjadi et al. 1991; Sajjadi and Pasquale, 1993; Pasquale, 1991). Extending the convention of designating the species of origin by the first letter, we refer to the rat homologs of the HEK receptors as Reks (rat EPH-like kinases).

HEK4 is the human homolog of the chicken receptor Cek4 (91% amino acid identity in the catalytic domain) and the mouse receptor Mek4 (96% amino acid identity in the catalytic domain). The amino acid sequence of HEK5 is very closely related (96% amino acid identity in the catalytic domain) to the chicken receptor Cek5 (Pasquale et al. J. Neuroscience 12, 3956-3967 (1992); Pasquale, 1991). HEK7 is probably the human homolog of the recently reported Cek7 (Sajjadi and Pasquale, 1993). HEK8 is likewise very closely related to Sek (Gilardi-Hebenstreit et al., 1992)) and Cek8 (95% amino acid identity in the catalytic domain) (Sajjadi

and Pasquale, 1993)). The human homologs for Cek6 and Cek9 have yet to be reported, while the human homolog of Cek10 has just recently been published. One of our human receptors has no close relatives in other species and apparently represents a novel member of the EPH sub-family. We have designated this receptor HEK11, assuming that human homologs for Cek 9 and 10 will be named HEK9 and HEK10, respectively. A summary of known EPH sub-family members is shown in Table 2.

TABLE 2

EPH receptor sub-family members

Human	Non-human homologs
EPH	None identified
ECK	None identified
None identified#	Eek
HEK4*	Cek4, Mek4
HEK5	Cek5, Nuk, ERK
None identified#	Cek6, Elk
HEK7	Cek7, Ehk1
HEK8	Cek8, Sek
None identified#	Cek9
HEK2	Cek10
HEK11	None identified
None identified	Ehk2

*published by Wicks et.al., 1992 as HEK

#Using the present nomenclature, the predicted human homolog of Eek is designated HEK3. For Cek6, the predicted human homolog is designated HEK6; For Cek9, the predicted human homolog is designated HEK9.

The predicted amino acid sequences of the four novel receptor clones and the previously known EPH sub-family members ECK (SEQ ID NO: 18), EPH (SEQ ID NO: 19), HEK2 (SEQ ID NO: 20) and HEK4 (SEQ ID NO: 21) were aligned as shown in Fig. 5. The four clones are closely related to each other and to the known EPH sub-family members. The extracellular domain sequences of all four novel receptors contain the Ig-loop, fibronectin-type III repeats, and cysteine-rich region characteristic of EPH sub-family members. The positions of the 20 cysteine residues are conserved among all sub-family members. Also completely conserved is the portion of the catalytic domain used as the basis for the EPH sub-family specific primer (Val-Cys-Lys-Val-Ser-Asp-Phe-Gly, SEQ ID NO: 2, amino acids 757-764 in Fig. 5). Table 3 summarizes the percentage of sequence identity between pairs of human EPH sub-family members. The lower portion of the table shows percent amino acid identity in the catalytic domain while the upper half shows percent amino acid identity in the extracellular region. The amino acid sequences of the EPH-like receptors are extremely well-conserved (60-89% amino acid identity) in the catalytic region but not as highly conserved in the extracellular region (38-65% amino acid identity), as would be expected for members of the same receptor sub-family.

TABLE 3

Eph family amino acid sequence comparison

extracellular domains

	EPH	ECK	HEK4	HEK5	HEK7	HEK8	HEK2	HEK11
EPH	*	47	42	38	40	43	40	42
ECK	62	*	47	41	45	46	41	46
HEK4	62	76	*	53	65	61	51	59
HEK5	60	74	81	*	52	53	63	51
HEK7	61	76	89	83	*	62	48	61
HEK8	62	76	86	85	88	*	52	57
HEK2	61	74	81	89	82	83	*	48
HEK11	60	74	83	83	85	85	80	*

Catalytic domains

Numbers shown are precent identity

Pairwise comparisons of amino acid sequences can be used to construct phylogenetic trees depicting the evolutionary relatedness of a family of molecules. Figure 6 is such a tree, which summarizes the relationships among the EPH sub-family members. Only one family member is shown from each group of cross-species homologs and the human representative was used whenever possible (refer to Table 2 for a summary of cross-species homologs). The branch lengths represent the degree of divergence between members. It has been shown previously that the EPH sub-family lies on a branch evolutionarily closer to the cytoplasmic PTKs than to other receptor PTKs (Lindberg and Hunter, 1993). Interestingly, the further one moves up the tree, the more closely related the receptors become and expression becomes more localized to the brain.

EXAMPLE 3

Construction and Expression of HEK Receptor Extracellular Domains

Soluble extracellular forms of HEK receptor proteins were constructed by deletion of DNA sequences encoding transmembrane and cytoplasmic domains of the receptors and introduction of a translation stop codon at the 3' end of the extracellular domain. A construct of the HEK5 extracellular domain had a stop codon introduced after lysine at position 524 as shown in Figure 1; the HEK7 extracellular domain was constructed with a stop codon after glutamine at position 547 as shown in Figure 2; the HEK 8 extracellular domain was constructed with a stop codon after threonine at position 547 as shown in Figure 3.

HEK extracellular domain was amplified from a human fetal brain cDNA library by PCR using primers 5' and 3' to the extracellular domain coding region.

For HEK5, the primers

5' CTGCTCGCCGCCGTGGAAGAAACG (SEQ ID NO: 22) and;
5' GCGTCTAGATTATCACTTCTCCTGGATGCTTGTCTGGTA (SEQ ID NO: 23)

were used to amplify the extracellular domain and to provide a restriction site for cloning into plasmid pDSRα. In addition, the following primers were used to provide a translational start site, the elk receptor signal peptide for expression; and a restriction site for cloning into pDSRα:

5' GCGGTCGACGCCGCCGCCATGGCCCTGGATTGCCTGCTGCTGTTCCTCCTG (SEQ ID NO: 24) and;

5' CGTTTCTTCCACGGCGGCGAGCAGAGATGCCAGGAGGAACAGCAGCAGGCA ATC (SEQ ID NO: 25)

The resulting construct resulted in fusion of DNA encoding the elk signal sequence Met-Ala-Leu-Asp-Cys-Leu-Leu-Leu-Phe-Leu-Leu-Ala-Ser (SEQ ID NO: 26) to the first codon of the HEK5 receptor.

The resulting HEK5 extracellular domain was cloned into pDSRα after digestion with SalI and XbaI and transfected into CHO cells for expression.

HEK8 extracellular domain was amplified from a human fetal brain cDNA library by PCR using primers 5' and 3' to the extracellular domain coding region. For HEK8, the primers

© 5' **GAATTCGTCGACCCGGCGAACCATGGCTGGGAT** and SEQ ID NO: 31

5' **GAATTCTCTAGATTATCATGTGGAGTTAGCCCCATCTC** SEQ ID NO: 32

were used to amplify the extracellular domain and to provide restriction sites for cloning into plasmid pDSRα.

The resulting HEK8 extracellular domain was cloned into pDSRα after digestion with SalI and XbaI and transferred CHO cells for expression.

HEK7 extracellular domain was amplified from a human fetal brain cDNA library by PCR using primers 5' and 3' to the extracellular domain coding region. For HEK7, the primers

© 5'**TTCGCCCTATTTTCGTGTCTCTTCGGGATTTGCGACGCTCTCCGGACCCTCCTG GCCAGC** and SEQ ID NO: 33

5' **GAATTCTCTAGATTATCACTGGCTTTGATCGCTGGAT** SEQ ID NO: 34

were used to amplify the extracellular domain. In addition, the following primers were used to provide a translational start site, the HEK8 receptor signal peptide sequence, and restriction site for cloning into plasmid pDSRα.

5'

GAATTCGTCGACCCGGCGAACCATGGCTGGGATTTTCTATTTCGCCCTATTTTCGT

GTCT SEQ ID NO. 35

5' **GAATTCTCTAGATTATCACTGGCTTTGATCGCTGGAT** SEQ ID NO. 36

The resulting construct resulted in fusion of DNA incoding HEK8 signal sequence **Met-Ala-Gly-Ile-Phe-Tyr-Phe-Ala-Leu-Phe-Ser-Cys-Leu-Phe-Gly-Ile-Cys-Asp** SEQ ID NO. 37 **to** the first codon of the HEK7 receptor.

The resulting HEK7 extracellular domain was cloned into pDSRα after digestion with SalI and XbaI and transfected into CHO cells for expression.

EXAMPLE 4

Antibodies to HEK Receptors

Antibodies to HEK receptor proteins were generated which recognize the extracellular domain by using bacterial fusion proteins as the antigen. Antibodies were also generated which recognize the cytoplasmic domain by using synthetic peptides as the antigen.

The methodology employed has been previously described (Harlow and Lane, In Antibodies: A Laboratory Manual, 1988). For the extracellular domain antibodies, cDNAs were inserted into the pATH vector (see Table 4 for the regions of each receptor encoded by this construct). These constructs were expressed in bacteria and the resultant TrpE-fusion proteins were purified by SDS-polyacrylamide gel electrophoresis. For the

cytoplasmic domain anti-peptide antibodies, peptides were synthesized (see Table 4 for the sequences) and covalently coupled to keyhole limpet hemocyanin . The fusion proteins and coupled peptides were used as antigens in rabbits and antisera were generated and characterized as described (Harlow and Lane, 1988). Anti-peptide antibodies were affinity purified by using a SulfoLink kit (Pierce, Rockford IL).

TABLE 4

HEK Receptor Antigens

Receptor	Peptide Sequences	Amino Acids in Fusion Protein
HEK4	[redacted] SEQ ID NO:38	22-159
HEK5	[redacted] SEQ ID NO:39	31-168
HEK7	[redacted] SEQ ID NO:40	335-545
HEK8	[redacted] SEQ ID NO:41	27-188
HEK11	[redacted] SEQ ID NO:42	187-503

EXAMPLE 5

HEK/TrkB Chimeric Receptors

1. Generation of pSJA1 encoding rat trkB cytoplasmic domain.

All of the chimeric receptors are composed of the extracellular domain and the transmembrane region of one of the HEK receptors and the intracellular portion of rat trkB. To simplify each individual construction, an intermediate or parental plasmid, called RtrkB/AflII (or pSJA1), was generated. First, without altering the coded peptide sequence, an AflII site (CTTAAG) was introduced into position 2021 (cytosine at position 2021

(C2021) to guanine at position 2026 (G2026, CTCAAG) of the rat trkB cDNA (Middlemas, et al., Mol. Cell. Biol. 11, 143-153 (1991)) by PCR aided mutagenesis. Briefly, PCR primers were synthesized based on the rat trkB cDNA sequence. Primer I encompassed C2003 to G2034 of the cDNA. This primer contained two mutations, a cytosine to thymine(T) substitution at position 2023 (C2023T) and an insertion of an adenine(A) in between T2013 and G2014. These mutations created the AflII site at position C2021 and an additional XhoI site flanking the AflII site. Primer II was in the reverse direction encompassing T2141 to A2165 of the cDNA which bore an ApaI site. The PCR fragment produced with these primers and the rat trkB cDNA template was digested with XhoI and ApaI enzymes and sub cloned into the XhoI and ApaI sites of an expression vector, pcDNA3 (InVitroGen), to generate pSJA1-b. Following, pSJA1-b was linearized with ApaI and ligated with a BanII digested rat trkB cDNA fragment (G2151 to G4697) to reconstitute a larger fragment (C2021 to G4697) including the coding sequence of the whole intracellular domain of the rat trkB protein (L442 to G790) and 1571 residues (A3131 to G4697) of the 1627 nucleotide 3'-end non-coding region of the cDNA.

2. Generation of HEK8/rat trkB (pSJA5) chimera.

HEK8/rat trkB chimera was generated with a similar strategy as mentioned above. A SalI/BsaI cDNA fragment was first isolated from plasmid TK10/FL13. This fragment included the nucleotide sequence from the beginning to T1689 of the HEK8 cDNA (Figure 3). Then, a pair of oligonucleotides was synthesized based on the HEK8 cDNA sequence. The sequence of the first oligonucleotide was the same as G1690 to C1740 of the Hek8 cDNA, with an additional C residue added to its 3'-end. The second oligonucleotide was in the reverse

orientation of the HEK8 cDNA. It contained C1694 to C1740 of the HEK8 cDNA sequence and an additional five residue motif, TTAAG, at its 5'-end. These two oligonucleotides were kinased and annealed with equal molar ratio, to create a double strand DNA fragment with the sequence of G1690 to C1740 of the HEK8 cDNA and with the BsaI and the AflII cohesive ends at its 5' and 3' ends, respectively. This fragment was ligated together with the SalI/BsaI cDNA fragment into XhoI/AflII linearized pSJA1 to generate the HEK8/RtrkB (pSJA5) chimerical construct.

3. Generation of HEK11/rat trkB (pSJA6) chimera.

To generate the HEK11/rat trkB chimera, a SalI/AccI fragment covering the sequence of nucleotide C1 to T1674 of the HEK11 cDNA (Figure 4) was first isolated from plasmid TK19T3. Then, a pair of oligonucleotides was synthesized based on the HEK11 cDNA sequence. The first oligonucleotide had the same sequence as from nucleotide A1666 to T1691 of the HEK11 cDNA, which contained the AccI site. The second oligonucleotide was in the reverse orientation of the HEK11 cDNA. It encompassed G1895 to T1919 of the HEK11 cDNA sequence. An additional ten residue motif, [redacted] SEQ ID NO: 43 was added to the 5'-end of this oligonucleotide to introduce an AflII site, which would be used to link the external domain and the transmembrane region of the HEK11 receptor to the intracellular domain of the rat trkB cDNA cloned in pSJA1 in the same reading frame. PCR was performed with these oligonucleotides as primers and the HEK11 cDNA as template. The PCR fragment was digested with AccI and AflII enzymes and ligated with the SalI/AccI cDNA fragment and the XhoI/AflII linearized pSJA1 to generate the HEK11/rat trkB (pSJA6) chimerical construct.

EXAMPLE 6

Tissue Distribution of HEK Receptors

The distribution of mRNA expression for HEK4, HEK5, HEK7, HEK8 and HEK11 receptors in human and rat tissues was examined by Northern blot hybridization.

Rat total RNA was prepared from tissues using the method of Chomczynski and Sacchi (Anal. Biochem 162, 156-159 (1987)). The RNA was separated by formaldehyde-agarose electrophoresis and transferred to Hybond-N membranes (Amersham, Arlington Heights, IL) using 20X SSC (Maniatis et al. 1982). The membrane was dried at 80°C in vacuo for 30 minutes, then crosslinked for 3 minutes on a UV transilluminator (Fotodyne, New Berlin, WI). The membrane was prehybridized for 2 hours at 42°C in 50% formamide, 5X SSPE, 5X Denhardt's, 0.2% SDS, and 100 µg/ml denatured herring sperm DNA (Maniatis et al. 1982). Northern blots of human tissue were purchased from Clontech (Palo Alto, CA). Probes were prepared by labeling the fragment of cDNA which encoded the extracellular domain of the receptor with ^{32}P-dCTP using a hexanucleotide random priming kit (Boehringer Mannheim, Indianapolis, IN) to a specific activity of at least $1x10^9$ cpm/ug. The probe was hybridized to the membrane at a concentration of 1-5 ng/ml at 42°C for 24 to 36 hours in a buffer similar to the prehybridization buffer except that 1X Denhardt's was used. After hybridization, the membranes were washed 2 times for 5 minutes each in 2X SSC, 0.1% SDS at room temperature followed by two 15 minute washes in 0.5X SSC, 0.1% SDS at 55°C. Blots were exposed for 1-2 weeks using Kodak XAR film (Kodak, Rochester, NY) with a Dupont Lightning Plus intensifying screen. The results are shown in Figures 7-11.

Homologs for HEK4 have been previously identified from mouse, chicken, and rat. In the adult mouse, expression is detected primarily in the brain and testis (Sajjadi et al. 1991). A slightly different pattern was found in adult chicken tissues, with the main sources of expression being the brain, liver, and kidney. Lower levels of expression were detectable in the lung and heart (Marcelle & Eichmann, Oncogene 7, 2479-2487 (1992)). A fragment of the *Rek4* gene (tyro-4) has been isolated and used to look at tissue expression in the adult rat (Sajjadi et al. 1991). The brain was the only tissue that expressed *Rek4* mRNA. However, RNA from lung or testis were not examined. Previous studies on *HEK4* only looked at the expression of the mRNA in cell lines, where it was found in one pre-B cell line and two T-cell lines (Wicks et al. 1992). The significance of this with regard to <u>in vivo</u> expression remains to be determined. In this study we have looked at the *HEK4* expression in human tissues, and also the expression of *Rek4* in rat tissues. The *HEK4* mRNA corresponds to a single transcript with a size of about 7 kb (Fig 7A). *HEK4* mRNA was most abundantly expressed in placenta, with lower levels present in heart, brain, lung, and liver. On prolonged exposures, trace amounts of mRNA were detectable in kidney and pancreas. Expression in the rat was more similar to that detected in the mouse and chicken. *Rek4* was expressed at the lowest levels of any of the family members characterized herein. A transcript of about 7 kb was detectable in rat lung, with a lower amount detectable in brain (Fig. 7B). Also, a 4 kb transcript was expressed in rat testis. Because the transcripts were barely detectable using total RNA, some of the other rat tissues may contain amounts of *Rek4* below the level of detection.

The expression of HEK5 in adult tissues has been previously studied in chicken and rat. Studies in the chicken have identified the Cek5 protein in the brain and liver, with a smaller protein detected in the intestine. In the rat, the tyro-5 fragment detected mRNA expression only in the adult brain, though intestine was not examined (Lai and Lemke, 1991). Our results show that *HEK5* mRNA was expressed at much higher levels than *HEK4* and was found as transcripts of several sizes. The most abundant mRNAs were of approximately 4.0 and 4.4 kb, with lesser amounts of higher molecular weight transcripts of 9.5 kb and longer (Fig. 8A). The *HEK5* mRNA was most abundantly expressed in placenta, but was also highly expressed in brain, pancreas, kidney, muscle, and lung. Longer exposures of the blots revealed the presence of transcripts in heart and liver as well. The rat homolog of *HEK5* (*Rek5*) showed a somewhat similar pattern of expression. *Rek5* was most abundant in intestine, followed by brain, kidney, lung, thymus, stomach, and ovary (Fig. 8B). Expression was not detectable in testis, muscle, heart, or liver. During our analysis of this family, we concluded that the rat *Erk* fragment (Chan & Watt, 1991) likely encodes a portion of the Rek5 receptor. *Erk* expression was examined in several rat tissues and found only in the lung. The reason for the discrepancy between that report and what we and others (Lai & Lemke, 1991) have found is unclear.

Homologs for *HEK8* have been identified from chicken, mouse, and rat. In the adult chicken, a single *Cek8* transcript was found to be expressed at high levels in the brain, with expression also d tected in the kidney, lung, muscle, and thymus. The expression of the mouse homolog of *HEK8*, *Sek*, has b en detected as a single transcript with abundant expression in th adult

brain and lower expression in the heart, lung and kidney. A fragment of *Rek8* (tyro-1) was used to look at expression in rat tissues, with expression found only in the brain (Lai & Lemke, 1991). We found that *HEK8* mRNA was expressed at levels comparable to that of *HEK5*. Multiple transcripts were also observed, the most abundant at 7 kb and 5 kb. The highest level of mRNA expression was seen in the brain, although substantial levels were detected in other tissues including heart, lung, muscle, kidney, placenta, and pancreas. Expression in liver was much lower than in the other tissues. The only difference in expression patterns between human and mouse was expression in human muscle, also seen for *Cek8* in chicken. Among the rat tissues, *Rek8* was most highly expressed in the brain, followed by the lung, heart, and testis (Fig. 10B). In contrast to *HEK8*, expression of *Rek8* appeared to be lower in muscle and kidney, two tissues where *HEK8* was readily detectable. In addition, *Rek8* was not expressed as a 5.0 kb transcript, as it was not visible even on prolonged exposures.

During the analysis of this family, we deduced that HEK7 is the human homolog of Cek7. The only expression seen in adult chicken was an 8.5 kb transcript found in the brain (Sajjadi & Pasquale, 1993). Of the five EPH sub-family members described here, *HEK7* was the most restricted in its expression pattern. Analysis of human mRNA revealed significant expression only in the brain, with a much lower level detectable in the placenta (Fig. 9A). Prolonged exposures did not reveal expression in any other tissue examined. Two prominent transcripts were found in brain, the most highly expressed with a siz of 6 kb and the other with a length of 9 kb. In the placenta, however, only the 9 kb transcript was detected. *Rek7*

mRNA was expressed in a pattern similar to *HEK7*. The highest level of expression was found in brain, with a much lower level in ovary (Fig. 9B). The transcripts were of similar size as for *HEK7*, with the 6 kb transcript detected only in brain.

HEK11 was expressed as several transcripts, with major mRNAs of length 7.5, 6.0 and 3.0 kb and minor transcripts of 4.4 and 2.4 kb (Fig. 11A). All five mRNAs were expressed at the highest levels in brain, followed by heart. Placenta, lung and kidney had significant amounts of four of the five transcripts, with lower expression seen in muscle. Pancreas had barely detectable amounts of *HEK11* mRNA, while liver had no detectable *HEK11* transcript. *Rek11* had a similar pattern of expression, with four transcripts (10, 7.5, 3.5 and 3.0 kb) detected in brain (Fig. 11B).

The relative level of mRNA expression for each of the five receptors in all tissues studied is summarized in Table 5.

TABLE 5

Tissue Distribution of HEK Receptors

Human	HEK4	HEK5	HEK7	HEK8	HEK11
Brain	++	++	++	+++	++
Heart	+	+	bd	++	+
Kidney	+	+	bd	+	+
Liver	+	+	bd	+	bd
Lung	+	+	bd	++	+
Muscle	+	+	bd	++	+
Pancreas	+	++	bd	+	bd
Placenta	+++	+++	bd	++	+

Rat	HEK4	HEK5	HEK7	HEK8	HEK11
Brain	+	++	+++	+++	++
Heart	bd	bd	bd	+	bd
Intestine	bd	+++	bd	bd	bd
Kidney	bd	++	bd	bd	bd
Liver	bd	bd	bd	bd	bd
Lung	+	+	bd	++	bd
Muscle	bd	bd	bd	bd	bd
Ovary	bd	+	+	bd	bd
Stomach	bd	+	bd	bd	bd
Testis	+	bd	bd	+	bd
Thymus	bd	+	bd	bd	bd

bd= below detection

The transcripts for *HEKs* 4,5,8, and 11 were rather widely distributed in human tissue while *HEK7* was specific for brain. Expression patterns between rat and human tissue were roughly comparable given that the rat blots were less sensitive due to the use of total RNA rather than polyA$^+$. As was found for the *Cek* mRNAs by Sajjadi and Pasquale (Sajjadi & Pasquale, 1993), often there were several different size transcripts detected for a single receptor. The size distribution of the transcripts appears to be both tissue and species specific. Previous work has shown that the smaller transcript of *Mek4* encodes a potentially secreted receptor (Sajjadi et al. 1991).

The following sections describe Materials and Methods used to carry out experiments described in Example 1.

Isolation, cloning and sequencing of HEK receptor cDNAs

Fragments containing a portion of the catalytic domain of EPH sub-family receptors were generated using a polymerase chain reaction (PCR) with disrupted phage from a human fetal brain cDNA library as a template. A 10µl aliquot of the cDNA library (Stratagene, La Jolla, CA) was treated at 70°C for 5 minutes to disrupt the phage particles, then cooled on wet ice. The disrupted phage were added to 10µl of 10X Taq polymerase buffer, 8ul of 2mM each dNTP, 100 picomoles of each primer, and 1.5 µl of Taq polymerase (Promega, Madison, WI) in a total volume of 100µl. The reaction was run for 35 cycles, each consisting of 1 minute at 96°C, 1 minute at 50°C, and 2 minutes at 72°C. A 5 minute, 72°C incubation was added at the end to ensur complete extension. The primers used were degenerate mixtures of oligonucleotides based on amino

acid sequences which are highly conserved among EPH sub-family members.

5'AGGGAATTCCAYCGNGAYYTNGCNGC' (SEQ ID NO: 27);
5'AGGGGATCCRWARSWCCANACRTC'(SEQ ID NO: 28).

The products of the PCR reaction were digested with EcoRI and BamHI and cloned into M13mp19 (Messing, Methods Enzymol. (1983)) for sequence analysis. The five clones which were identified as fragments of EPH receptor sub-family members were labeled with ^{32}P-dCTP by random priming and each was used to screen Genescreen nitrocellulose filters (NEN, Boston, MA) containing plaques from the human fetal brain cDNA library. Phage stocks prepared from positively screening plaques were plated and rescreened with the same probe in order to obtain single clones. cDNA inserts were transferred into pBluescript using the in vivo excision protocol supplied with the cDNA library (Stratagene, La Jolla, CA). Nucleotide sequences were determined using Taq DyeDeoxy Terminator Cycle Sequencing kits and an Applied Biosystems 373A automated DNA sequencer (Applied Biosystems, Foster City, CA).

5' Race

The 5' ends of the cDNAs were isolated using a 5' RACE kit (GIBCO/BRL, Gaithersburg, MD) following the manufacturer's instructions. Excess primers were removed after first strand cDNA synthesis using ultrafree-MC cellulose filters (30,000 molecular weight cutoff, Millipore, Bedford, MA). Amplified PCR products were digest d with the appropriate restriction enzymes, separated by agarose gel electrophoresis, and purified using a Gen clean kit (Bio101, La Jolla, CA). Th purified PCR product was ligated into the plasmid vector pUC19 (Yanisch-Perron et al. Gene 33, 103-119 (1985))

which had been digested with appropriate restriction enzymes and the ligation mixture was introduced into host bacteria by electroporation. Plasmid DNA was prepared from the resulting colonies. Those clones with the largest inserts were selected for DNA sequencing.

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

(C) Nucl sequence listing (8/7/97)